Temperature-Sensitive Mutants in Herpes Simplex Virus Type 1 ICP4 Permissive for Early Gene Expression

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A large number of temperature-sensitive (*ts*) mutants of herpes simplex virus type 1 (HSV-1) in the gene encoding the immediate-early transcriptional regulatory protein, ICP4, have been isolated and characterized with respect to expression of the immediate-early, early, and late viral gene products. The hallmark of these mutants is the overproduction of immediate-early gene products and the underproduction of early and late gene products. The present study involves the preliminary genetic and molecular characterization of two unique regulatory mutants of HSV-1, *ts*48 and *ts*303. Genetically, both mutants exhibit inefficient complementation with eight *ts* mutants in complementation group 1-2, which defines the gene for ICP4, and marker rescue experiments place the mutations in both mutants in the 3' portion of the coding sequence for ICP4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of *ts*48- and *ts*303-infected cell polypeptides synthesized at the nonpermissive temperature demonstrates that immediate-early polypeptides ICP4 and ICP27 are overproduced, with the simultaneous production of early polypeptides ICP6, ICP8, gB, and others. Immediate-early polypeptides are resynthesized upon temperature shift-up early in infection; however, shift-up late in infection does not result in the resynthesis of immediate-early polypeptides. Late gene products are either absent or underrepresented under long-term labeling conditions. To examine the effects of the mutations in *ts*48, *ts*303, and other ICP4 mutants specifically on early gene expression, *trans*-induction experiments were performed in cells transfected with the gene for chloramphenicol acetyltransferase under early gene control (*tk*) and superinfected with KOS, *ts*B32, *ts*48, and *ts*303. Mutant *ts*B32 did not induce chloramphenicol acetyltransferase activity above the basal level; however, *ts*48 and *ts*303 induced chloramphenicol acetyltransferase activity nearly equal to wild-type levels. Fifteen to fifty percent of wild-type levels of viral DNA are synthesized at the nonpermissive temperature in *ts*48- and *ts*303-infected cells, indicating that immediate-early and early gene functions are intact (or nearly so) and that the block in *ts*48 and *ts*303 is in a regulatory event subsequent to that exhibited by other mutants in complementation group 1-2 which are DNA⁻.

The first viral gene products synthesized in herpes simplex virus type 1 (HSV-1) infection are five polypeptides: ICP4, ICP0, ICP22, ICP27, and ICP47 (15, 28, 33). These polypeptides, termed immediate-early or α polypeptides, are operationally defined as those encoded by genes which are transcribed and translated in the absence of prior viral protein synthesis (15, 16, 18). Functional immediate-early polypeptides are required for the expression of two subsequent kinetic classes of viral genes: the early and late genes. It has been shown that the requirement for immediate-early polypeptides is at the level of transcription (16, 18, 30, 31, 37).

The isolation and phenotypic characterization of temperature-sensitive (*ts*) mutants of HSV-1 has shed considerable light on the specific gene products and molecular mechanisms involved in the regulation of HSV gene expression. The *ts* mutations of mutants belonging to complementation group 1-2 have been mapped physically by marker rescue to DNA sequences within the short repeats of the HSV-1 genome (9, 32). These sequences are coincident with those encoding the immediate-early polypeptide ICP4 (molecular mass, 175 kilodaltons) (24, 26). Thus, the mutants in group 1-2 are defective in the structural gene for this polypeptide (9, 32). The hallmark of *ts* mutants in complementation group 1-2 is the dramatic overproduction of immediate-early polypeptides (6, 9, 30, 32) and mRNA (31, 37) and the concomitant underproduction of early and late polypeptides at 39°C, the nonpermissive temperature (6, 9, 30, 32). The mutants do not induce the synthesis of viral DNA at 39°C (1) because at this temperature they fail to express the early gene products required for viral DNA synthesis. Functional ICP4 is required only transiently for DNA synthesis and, therefore, is probably not directly involved in the DNA synthetic process (35). The observation that functional ICP4 controls the levels of expression of immediate-early proteins in the infected cell and is essential for transcription of both early and late genes implicates ICP4-associated function(s) throughout all phases of the HSV-1 replicative cycle (9, 30, 31, 37).

Only two eucaryotic proteins have been associated unequivocally with the positive regulation of gene expression; the E1a protein of adenovirus (17) and ICP4. Recently both have been shown to be capable of substituting for *cis*-acting enhancer elements 5' to the mRNA start sites of purified eucaryotic genes (14). In the HSV-1 system, ICP4 has been shown to act in *trans* to stimulate expression of the HSV-1 thymidine kinase (*tk*) gene resident in biochemically transformed cells (19, 21, 29). The target for the ICP4-mediated stimulation of transcription has been shown to be located immediately 5' to the *tk* gene (29).

How, specifically, ICP4 mediates the sequential and coordinate regulation of immediate-early, early, and late genes is unknown. Herein we report the preliminary genetic and phenotypic characterization of two *ts* mutants whose mutations map within the coding sequences for ICP4. Unlike previously characterized *ts* mutants in ICP4, however, both mutants induced nearly wild-type levels of early polypeptides and moderate levels of viral DNA at the nonpermissive

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temperature. Late proteins, however, were not detected. The genetic and phenotypic properties of these mutants suggest that ICP4 may act differentially on early and late gene expression.

MATERIALS AND METHODS

Virus and cells. Procedures for the growth and maintenance of Vero, CV-1, human embryonic lung (HEL), and primary rabbit kidney (RK) cells were conducted as described previously except that 10% fetal bovine serum was used (9, 39). The KOS strain of HSV-1 and the *ts* mutants used in this study were propagated as described (36). Mutants *ts*48 and *ts*303, the subjects of this investigation, were derived from strain KOS as described below. Mutant *ts*D, derived from HSV-1 strain 17 (4), was kindly provided by J. H. Subak-Sharpe (Institute of Virology, Glasgow, Scotland). Mutants *ts*B2, *ts*B21, *ts*B27, *ts*B28, *ts*B32, *ts*A24, and *ts*A42 were derived from strain KOS (5, 9, 34, 39).

Plasmids and bacteria. Recombinant plasmids were propagated in *Escherichia coli* HB101 by standard procedures (8). pSG28 was obtained from Rozanne Sandri-Goldin (University of Michigan, Ann Arbor, Mich.) and contains *Eco*RI fragment EK (map coordinates 0.724 to 0.866) in pBR325 (11). pKEB-X2 contains KOS DNA sequences between the *Xho*I site at map coordinate 0.806 and the *Eco*RI site at map coordinate 0.866 and was obtained from Craig Bond, (Pennsylvania State University, University Park, Pa.). The plasmids pKBK, pKBZ, and pKBK-S2 were subcloned from pKEB-X2 by standard techniques. For the locations of the viral DNA inserts of the clones used in this study with respect to the HSV-1 KOS genome and the gene for ICP4, see Fig. 2.

Genetic analysis. Complementation analysis was performed as described previously (34). Marker rescue experiments were performed by transfection of Vero or RK cells with coprecipitates of *ts* mutant-infected cell DNA and linearized recombinant plasmids containing wild-type viral DNA fragments. Mutant infected-cell DNA (7 to 10 µg), high-molecular-weight salmon sperm DNA (5 µg), and linearized plasmid DNA (1 to 3 µg) were coprecipitated with CaCl$_2$ (13). The precipitate was used to transfect RK or Vero cells as described by Parris et al. (27). Transfected cultures were then incubated at 34°C and progeny virus was assayed at 34 and 39°C to identify *ts$^+$* recombinants.

DNA isolation. Analytical and preparative quantities of bacterial plasmid DNA were isolated by the method of Birnboim and Doly (2). Preparative quantities were further purified by banding in CsCl.

For the preparation of infected-cell DNA, ca. 2 × 10^8 HEL cells were infected with virus at a multiplicity of infection (m.o.i.) of 0.01 PFU per cell. When cytopathic effects were generalized, cells were scraped into medium, pelleted, and resuspended in a small volume of TE buffer (10 mM Tris-hydrochloride [pH 8.0], 1 mM EDTA). Extracellular virus was pelleted and added to the cell pellet. The combined pellets were lysed with 0.5% sodium dodecyl sulfate (SDS) and digested for 4 h with 200 µg of proteinase K per ml at 37°C. The DNA was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). The DNA was then exhaustively dialyzed against cold TE.

Determination of viral DNA phenotype. Viral DNA phenotypes of *ts* mutants were determined as described by Aron et al. (1).

Gel electrophoresis. (i) Viral DNA. Restriction fragments of

viral DNA were separated by electrophoresis in agarose gels as described by Bolivar et al. (3).

(ii) Viral polypeptides. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of radioactively labeled infected-cell lysates was performed by the method of Laemmli (20), as modified by Manservigi et al. (23). Proteins and glycoproteins were labeled at the indicated times and temperatures with [^{35}S]methionine (10 µCi/ml) or [^{3}H]mannose (50 µCi/ml) (Amersham Corp., Arlington Heights, Ill.), respectively.

CAT assays. The procedure for the introduction of chloramphenicol acetyltransferase (CAT) plasmids into low-passage CV-1 cells and the assay for CAT in cell extracts was performed as described by Gorman et al. (12).
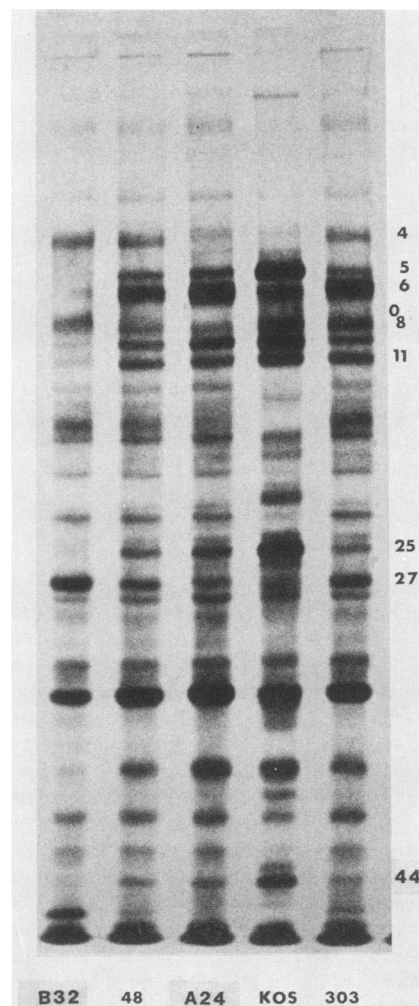

FIG. 1. Polypeptide phenotypes of *ts*48 and *ts*303. Confluent monolayers of HEL cells were infected with each virus at an m.o.i. of 20 PFU per cell. Adsorption for 1 h and subsequent incubation were carried out at 39°C. At 5 h postinfection (4 h postadsorption), 10 µCi of [^{35}S]methionine per ml was added to each culture. At 15 h postinfection, infected cell cultures were washed with ice-cold saline and directly solubilized in SDS-PAGE sample solution. Samples were then heated at 97°C for 5 min, loaded onto a 9% *N-N'*-diallyltartardiamide-cross-linked polyacrylamide gel, and electrophoresed for 17 h at 100 V. After electrophoresis, the gel was fixed in methanol-water-acetic acid (5:5:1), equilibrated with water, Autofluor (National Diagnostics, Somerville, N.J.), dried, and exposed to XAR-5 X-ray film.

RESULTS

Isolation of _ts_48 and _ts_303. Mutant _ts_48 was isolated after cotransfection of infectious KOS DNA with a cloned fragment of KOS DNA which has been subjected to in vitro mutagenesis with hydroxylamine. As is often the case, the mutation was not located within the region of the genome corresponding to the mutagenized fragment. Therefore, the mutation in _ts_48 either occurred spontaneously or was induced as a result of the transfection process. Mutant _ts_303 (_ts_L14) was generated by nitrosoguanidine mutagenesis of replicating KOS virus (34).

Polypeptide phenotypes of _ts_48 and _ts_303. Polypeptide synthesis in _ts_303-infected cells labeled from 4 to 18 h postinfection at the nonpermissive temperature was examined previously (6). In these early tests, it was noted that the immediate-early polypeptides were overproduced, with the simultaneous production of significant levels of early polypeptides. We confirmed the results of the earlier studies of ts303 and showed that the polypeptide profile of _ts_48-infected cells is similar (Fig. 1). Thus, in both _ts_48- and _ts_303-infected cells at 39°C, the immediate-early polypeptides ICP4, ICP0, and ICP27 were overproduced. Notably, ICP4 was overproduced to the same extent as in _ts_B32-infected cells. Mutant _ts_B32 is a previously characterized member of complementation group 1-2, the group which defines the gene for the immediate-early polypeptide, ICP4. All mutants in complementation group 1-2 isolated to date induce the overproduction of immediate-early polypeptides, yet these mutants induce little or no early or late polypeptide synthesis (9, 32). By contrast, _ts_48 and _ts_303 induce significant levels of both early (β) and delayed-early (β-γ) polypeptides, as indicated by the production of ICP5, ICP6, ICP8, ICP11, ICP25, ICP39, and ICP44 (Fig. 1). With the exception of the overproduction of the immediate-early polypeptides ICP4 and ICP27, the polypeptide profiles of cells infected at 39°C with _ts_48 and _ts_303 appear similar to that of _ts_A24-infected cells. Mutant _ts_A24 is defective in the early gene encoding ICP8 (39), the major HSV DNA-binding protein required for viral DNA synthesis (22). The overproduction of immediate-early polypeptides, with the simultaneous production of early polypeptides in the absence of late polypeptide synthesis, presents an interesting problem with regard to the regulation of gene expression. To date, the only instances in which the overproduction of immediate-early polypeptides have been observed are (i) in cells infected at 39°C with _ts_ mutants in the gene for ICP4 (30, 31) and (ii) wild-type-virus-infected cells in the presence of inhibitors of protein and RNA synthesis (15). In that _ts_ mutants in ICP4

TABLE 1. Results of complementation tests among nine HSV-1 _ts_ mutants

Mutant	_ts_303	_ts_D	_ts_B2	_ts_B21	_ts_B27	_ts_B32	_ts_B28	_ts_A24	_ts_A42
_ts_48	0.8[a]	0.5	0.9	3.9[a]	0.5	0.4	0.4	102[a]	182
_ts_303		0.1	0.3	0.1	0.1	0.5	0.1	56	50
_ts_D			1.1	1.2	0.8	1.2	1.6	47	ND[b]
_ts_B2				0.6	1.6	0.3	1.0	34	ND
_ts_B21					0.9	0.4	0.4	59	ND
_ts_B27						0.4	0.6	43	ND
_ts_B32							0.6	108	ND
_ts_B28								42	ND
_ts_A24									ND

[a] Complementation indices of <2 are considered to be negative; indices between 2 and 10 may reflect intragenic complementation; values of 10 or greater are considered to be positive.
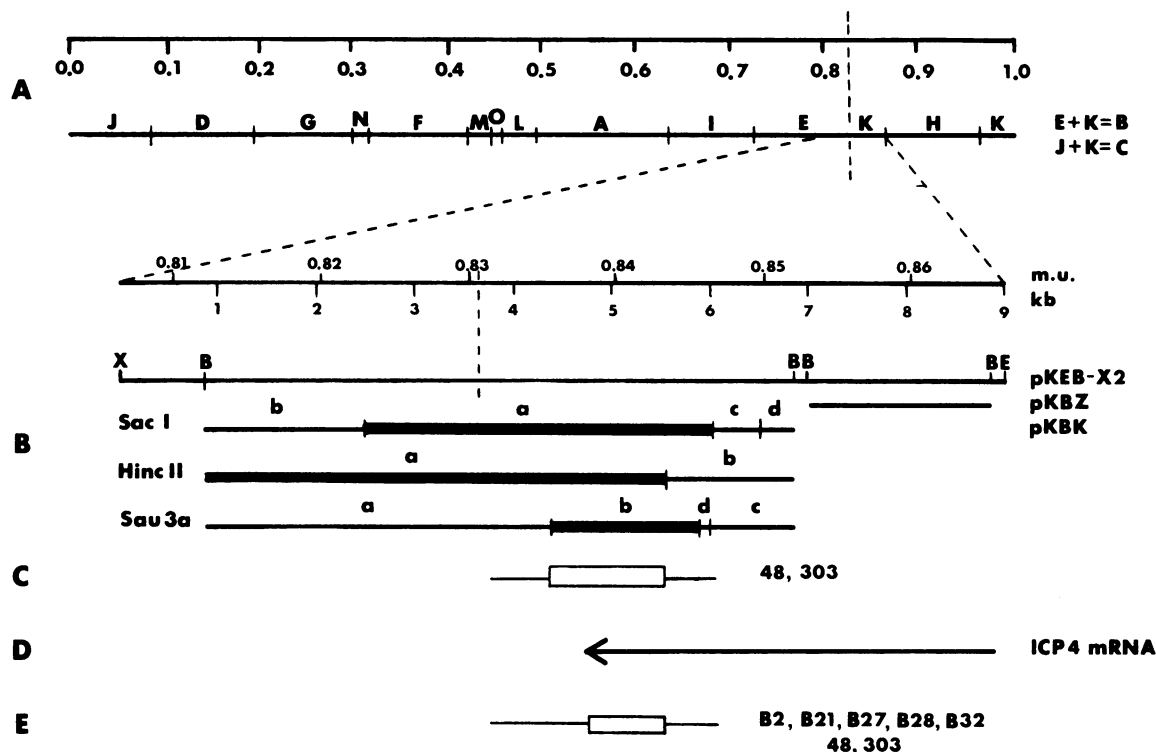[b] ND, Not done.

TABLE 2. Marker rescue of _ts_ mutations in _ts_48 and _ts_303 by viral DNA fragments Between coordinates 0.724 and 0.866

Fragment (clone)	Coordinates	Rescue[a]	
		_ts_48	_ts_303
_Eco_RI-EK (pSG28)	0.724–0.866	23	3.0
(pKEB-X2)[b]	0.806–0.866	26	66
_Bam_HI-Z[c] (pKBZ)	0.853–0.865	<0.005	ND[f]
_Bam_HI-K (pKBK)	0.812–0.852	47	47
_Sac_I-a[d]	0.823–0.846	0.9	ND
_Sac_I-b[d]	0.812–0.823	<0.005	ND
_Sac_I-c[d]	0.846–0.850	<0.005	ND
_Sac_I-d[d]	0.850–0.852	<0.005	ND
_Hinc_II-a[d]	0.812–0.843	1.0	2.0
_Hinc_II-b[d]	0.843–0.852	<0.01	<0.02
_Sau_3A-b[e] (pKBK-S2)	0.835–0.845	0.6	2.1
No fragment		<0.005	<0.005

[a] Values for marker rescue were taken as the titer of the progeny of transfection assayed at 39°C divided by the titer at 34°C ($\times 10^3$).
[b] See Fig. 2.
[c] _Bam_HI fragment Z (1.84 kilobases) of KOS corresponds to _Bam_HI fragment Y (1.84 kilobases) of other HSV-1 strains.
[d] These fragments were eluted from agarose gels after the appropriate digestion of pKBK.
[e] _Sau_3A subfragment b of pKBK was cloned into the _Bam_HI site of pBR325.
[f] ND, Not done.

induce little or no early and late polypeptide synthesis, _ts_48 and _ts_303 appear to represent a separation of these two phenotypes.

Genetic characterization of _ts_48 and _ts_303. (i) Complementation. Mutant _ts_303 had previously been shown to complement nearly all mutants in the standard set of KOS mutants available at the time (34). Hence, _ts_303 was designated _ts_L14 to signify that it defined an unique complementation group. In this previous study, however, very inefficient complementation (complementation index, <5) was observed between _ts_303 and the two members of complementation group 1-2, _ts_B2 and _ts_B21. At the time these studies were conducted, a complementation index of 2 or greater was taken to indicate complementation, i.e., that the two mutants were in different genes. More recently it has become clear that HSV-1 mutants in the same gene can exhibit intragenic complementation, yielding indices ranging from 2 to 10 (38). In light of these more recent findings, it became desirable to retest the levels of complementation obtained with _ts_303 and a variety of mutants in group 1-2, as well as with _ts_48 (Table 1). Mutants _ts_B2, _ts_B21, _ts_B27, _ts_B28, _ts_B32, and _ts_D are members of complementation group 1-2 (5, 34, 36). Mutants _ts_A24 and _ts_A42 are members of complementation group 1-1, which defines the gene for ICP8 (39). Based on the results of the complementation tests presented in Table 1 and other complementation tests (data not shown), several points can be made. (i) Mutants _ts_48 and _ts_303 fail to complement each other and hence are in the same complementation group. (ii) Both _ts_48 and _ts_303 failed to complement members of complementation group 1-2. The low level of complementation observed with _ts_B21 and _ts_48 is reproducible. Likewise, low levels of complementation between _ts_B21 and _ts_303 are also observed occasionally (complementation index, 2 through 10; unpublished observations), although no complementation was detected in the test whose results are shown in Table 1. (iii) Mutants _ts_48 and _ts_303 complemented mutants in another complementation group (group 1-1) efficiently. Taken together, the complementation data suggest that _ts_48 and _ts_303 contain mutations in ICP4. The less restrictive phenotype of these mutants compared with other

FIG. 2. Physical map locations of *ts* mutations in *ts*48 and *ts*303. (A) The *Eco*RI restriction map of HSV-1 strain KOS is shown in the prototypic orientation of the genome beneath the scale of map coordinates. (B) The *Xho*I-*Eco*RI fragment between map coordinates 0.806 and 0.866 is expanded and illustrates the locations of fragments used for marker rescue of *ts*48 and *ts*303. The *Sac*I and *Hinc*II fragments were gel purified from pKBK, digested with *Bam*HI-*Sac*I and *Bam*HI-*Hinc*II, respectively. *Sau*3A fragment b is contained in the plasmid pKBK-S2. The fragments with which positive marker rescue was demonstrated (Table 2) are indicated by heavy lines. X, *Xho*I; B, *Bam*HI; E, *Eco*RI. M. U., map units; Kb; kilobases. (C) The region of overlap between *Hinc*II fragment a and *Sau*3A fragment b defines the sequences containing the *ts* mutations in *ts*48 and *ts*303 (coordinates 0.835 to 0.843). (D) The location of the mRNA for ICP4 is also shown with respect to this region of the genome (7). (E) Shown is the inferred location of the *ts* mutations in KOS mutants in complementation group 1-2.

ICP4 mutants suggests a differential involvement of ICP4 during the course of lytic infection.

(ii) **Physical mapping.** The results of the complementation tests shown in Table 1 suggested that the *ts* lesions in *ts*48 and *ts*303 were associated with the gene for ICP4. To determine the specific locations of the *ts* mutations in *ts*48 and *ts*303 on the HSV-1 genome, marker rescue experiments were performed. Marker rescue experiments with cloned KOS DNA fragments confirmed this suggestion (Table 2; Fig. 2). The rescue frequencies for the various fragments tested are presented in Table 2, and these data are summarized in Fig. 2. The smallest fragment which rescued both mutations was *Sau*3A fragment b (map coordinates 0.835 to 0.845). However, because *Hinc*II fragment a also rescued both mutations, the limits of the smallest sequence able to rescue are map coordinates 0.835 to 0.843. Moreover, because the 3' terminus of the ICP4 message spans the 0.837 to 0.843 sequences (7), the mutations in *ts*48 and *ts*303 must lie within this 700-base pair sequence. It is notable that the mutations in *ts*48 and *ts*303, like all other KOS mutants in complementation group 1-2, lie within this region—regardless of the method of mutagenesis used in their induction.

Detailed phenotypic characterization of *ts*48 and *ts*303. (i) Synthesis of viral proteins and glycoproteins. As shown previously, other mutants whose *ts* mutations lie in ICP4 (i.e., *ts*B32) do not express early or delayed-early genes to the same extent as do *ts*48 and *ts*303 (Fig. 1) (9). To

determine whether the differences in the polypeptide phenotypes of *ts*48 and *ts*303 relative to other mutants in group 1-2 was a consequence of a greater degree of "leakiness," *ts*48- and *ts*B32-infected cells were labeled with [^{35}S]methionine at three temperatures: 34.0, 39.0, and 39.6°C from 5 to 15 h postinfection, and samples were taken for SDS-PAGE analysis and for infectivity assays.

The gel profiles shown in Fig. 3 confirm the difference in polypeptide synthesis between the two classes of ICP4 mutants as shown previously (Fig. 1). Mutant *ts*B32 was considerably more restricted with respect to the synthesis of early and late polypeptides than was *ts*48. Significant expression of early and delayed-early polypeptides (e.g., ICP5, ICP8, ICP11, and ICP25) occurred in *ts*48-infected cells as both elevated temperatures, whereas late polypeptides (e.g., ICP1, ICP2, ICP15, ICP19, ICP20, and ICP43) were not produced in these cells at either nonpermissive temperature. Importantly, based on the levels of infectious mutant virus produced, both 39.0 and 39.6°C proved to be equally nonpermissive for the growth of *ts*B32 and *ts*48 (Table 3; see the legend to Fig. 3).

To further substantiate the absence of late gene expression in mutant-infected cells, cells were labeled with [^{3}H]mannose (Fig. 4), and the levels of viral glycoprotein synthesis were assessed. Glycoprotein gC is a member of the late class of HSV-1 polypeptides (10), whereas gB is a member of the early class with respect to the times of onset and maximum
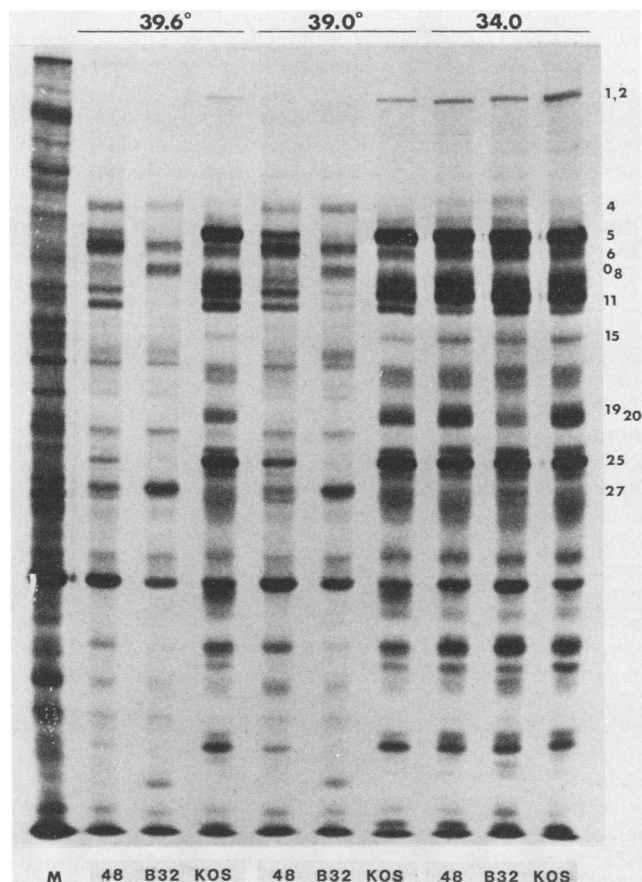
FIG. 3. Polypeptides synthesized in *ts*48-, *ts*B32-, and KOS-infected cells at 34, 39.0, and 39.6°C. See the legend to Fig. 1 for experimental details. Replicate cultures were harvested and assayed for infectious virus at all three temperatures. An uninfected culture was labeled and prepared in the same manner (lane M). The yields relative to KOS for *ts*B32 and *ts*48 at 39.0°C were 2.2×10^{-3} and 3.9×10^{-3}, respectively. At 39.6°C, the relative yields of *ts*B32 and *ts*48 were 1.7×10^{-3} and 2.9×10^{-3}, respectively. Revertants were not observed when the assays were conducted at 39 and 39.6°C.

TABLE 3. Growth characteristics of mutants under nonpermissive conditions

Virus	Yield[a] at 39°C (PFU/ml)	Plating efficiency[b]	
		39.0°C	39.6°C
KOS	1×10^7	0.8	0.6
*ts*D	8×10^1	ND[c]	ND
*ts*B2	9×10^1	ND	ND
*ts*B21	5×10^0	ND	ND
*ts*B27	1×10^3	ND	ND
*ts*B32	4×10^2	2×10^{-6}	2×10^{-6}
*ts*B28	6×10^1	ND	ND
*ts*A24	3×10^2	ND	ND
*ts*303	5×10^2	5×10^{-7}	5×10^{-7}
*ts*48	7×10^1	10^{-5}	2×10^{-6}

[a] 2.0×10^5 Vero cells were infected at a m.o.i. of 2.5. Cultures were incubated at 39.0°C for 18 h.
[b] PFU per milliliter at 39.0°C or 39.6°C (nonpermissive)/PFU per milliliter at 34°C (permissive).
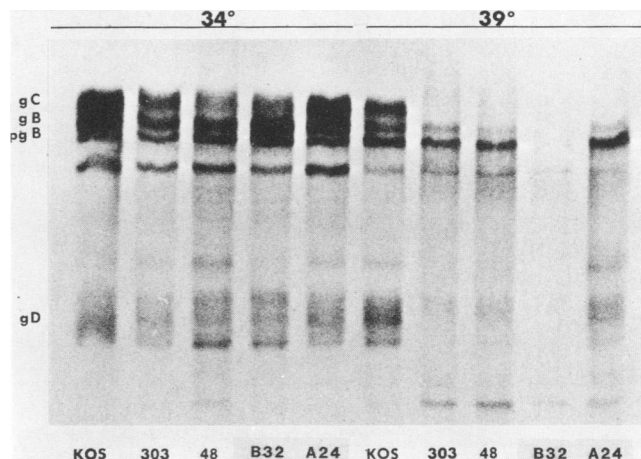[c] ND, Not done.

FIG. 4. Glycoprotein phenotypes of *ts*48 and *ts*303 at 39.0°C. This experiment was conducted with the indicated viruses essentially as described in the legend to Fig. 1, except that 50 μCi of [³H]mannose per ml was used as label.

rates of their synthesis. Consistent with the profiles of polypeptide synthesis presented in Fig. 1 and 3, substantial quantities of pgB and gB and little or no gC were synthesized in cells infected with *ts*48 and *ts*303. The glycoprotein profiles of *ts*48- and *ts*303-infected cell extracts are similar to that of the *ts*A24 extract; *ts*A24 is blocked at a point between early and late gene expression. By contrast, *ts*B32 induced very low but detectable amounts of pgB and gB at the nonpermissive temperature. The difference in the observed levels of individual glycoproteins induced by *ts*B32 as compared with *ts*48 and *ts*303 is an especially clear reflection of the differences in the patterns of gene expression characteristic of these two groups of ICP4 mutants (*ts*B32 and others versus *ts*48 and *ts*303).

Importantly, this difference is not a consequence of the leak of *ts*48 and *ts*303 at the nonpermissive temperature, as the yields of infectious *ts*48 and *ts*303 at 39.0°C (Table 3) and 39.6°C (see the legend to Fig. 3) were of the same magnitude as the great majority of other *ts* mutants of strain KOS, including *ts*B32. In addition, the plating efficiencies of *ts*48, *ts*303, and *ts*B32 at 39 and 39.6°C are similar. Moreover, the mutation specifying the temperature sensitivity of *ts*48 is most probably responsible for the aberrant regulatory phenotype observed at the nonpermissive temperature. This is so because *ts*⁺ recombinants S1 and S2 isolated from the progeny of the transfection of *ts*48 DNA with the KOS *Sau*3A fragment (map coordinates 0.835 to 0.845) shown in Fig. 2 were wild type with respect to the synthesis of HSV-1 polypeptides at 39°C (Fig. 5).

(ii) **Viral DNA synthesis.** The HSV-1-specific enzymes involved directly in the synthesis of viral DNA belong to the early class of viral polypeptides (1). Mutant *ts*B32, as well as all previously described *ts* mutants in ICP4, do not induce the synthesis of viral DNA because they fail to express all early proteins at the nonpermissive temperature.

At the nonpermissive temperature, nearly wild-type levels of wild-type early polypeptides are synthesized in *ts*48- and *ts*303-infected cells; therefore, one might expect detectable levels of viral DNA to be synthesized. The CsCl gradient profiles of DNA synthesized in *ts*48- and *ts*303-infected cells (Fig. 6) are consistent with this expectation. Thus, in *ts*48- and *ts*303-infected cells at 39°C, 21 and 41%, respectively, of the wild-type level of viral DNA was synthesized. No viral
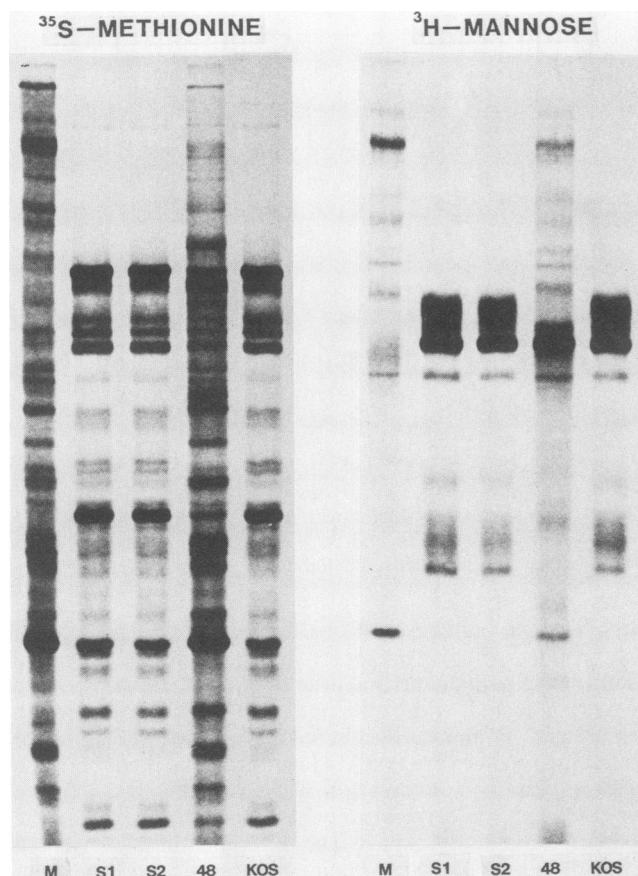
FIG. 5. Polypeptide phenotypes of ts^+ recombinants of ts48. Two ts^+ recombinants of ts48 (S1 and S2) were isolated from a marker rescue experiment with pKBK-S2 (Sau3A; Fig. 2 and Table 2). Extracts for SDS-PAGE analysis were prepared from cells infected with the ts^+ recombinants (S1, S2), ts48, and KOS. Infected cells were incubated at 39.4°C and labeled from 5 to 15 h postinfection with either [^{35}S]methionine or [^{3}H]mannose as indicated. Extracts from uninfected cells were also prepared (lane M). The elevated temperature (39.4°C) was used in this test to confirm the fully wild-type character of S1 and S2.

DNA was detected in tsB32-infected cells at this temperature.

The decreased level of viral DNA synthesis observed in ts48-infected cells relative to that seen in ts303-infected cells may be due to the lower levels of ICP8 present in these cells than in ts303- and KOS-infected cells (see Fig. 3). Wild-type levels of ICP8 were observed in ts48-infected cells at 39.6°C after short pulses of [^{35}S]methionine, and the level of ICP8 observed did not diminish with respect to wild-type virus-infected cells in a subsequent chase (data not shown). The expression of ICP8 in ts48-infected cells above 39°C after reversal of a cyclohexamide block, however, was decreased with respect to other early polypeptides (data not shown). These observations indicate that the mRNA for ICP8 may be unstable in ts48-infected cells at 39°C.

Relative to KOS, the mutants tsB32, ts48, and ts303 all failed to induce the expression of an activity which is required for the efficient shut-off of host-cell protein synthesis (Fig. 1). The shut-off of cellular DNA synthesis was also compromised in cells infected with each of these three mutants (Fig. 6).

Effects of ts mutations in ICP4 on early promoter activity. It

may be hypothesized that the phenotypes of ts48 and ts303 are due to the ability of the mutant ICP4 to have a positive regulatory effect on early but not late promoters. However, because different levels of viral DNA were synthesized in KOS-, tsB32-, ts48-, and ts303-infected cells at 39°C (Fig. 6) and hence the number of transcribable templates varied considerably, it was difficult to assess directly the activity of promoters for the different kinetic classes of genes. Therefore, the BglII-BamHI fragment at the right-hand terminus of BamHI-P containing the early promoter for the thymidine kinase gene (25) was fused to the 5' leader sequence of the CAT gene in pSV2Cat (12) to generate the plasmid pTKCat (Fig. 7). Upon transfection of CV-1 cells with CAT plasmids, the CAT activity induced is a direct measure of expression from the promoter fused to the CAT gene (12). The level of CAT activity is reflected by the degree of acetylation of the input chloramphenicol. Replicate monolayers of CV-1 cells were transfected with pTKCat and 3 h later were infected with the indicated viruses at 39°C. Cultures were harvested 15 h later, and CAT activity was measured. Extracts of KOS-, ts48-, and ts303-infected cells contained greatly elevated levels of CAT activity compared with those observed in uninfected cells (Fig. 7, lane M) and in tsB32-infected cells. The increase in CAT activity in KOS-, ts48-, and ts303-infected cells above that in uninfected cells is a result of $trans$-activation of the tk promoter directing the synthesis of CAT mRNA. Since the pTKCat construct does not contain a eucaryotic origin of replication, the number of transcribable templates is limited and controlled by the input DNA. Therefore the effects of mutant forms of ICP4 on early tk promoter activity can be directly compared with tk promoter activity in wild-type infected and uninfected cells. In this case, tk promoter activity in ts48- and ts303-infected cells was nearly equal to that of wild-type virus. By contrast, in tsB32-infected cells, little activity was detected. This observation indicates that the elevated levels of early polypeptides synthesized by ts48 and ts303 relative to tsB32 is most probably due to elevated levels of early mRNA. Furthermore, this increase is mediated by the transcriptional control region 5' to the tk gene (Fig. 7).

Temperature-shift experiment. A common property of all ts mutants in ICP4 isolated to date is the overproduction of immediate-early polypeptides at 39°C (9, 30). This observation led to the conclusion that among its essential functions, ICP4 is also involved—directly or indirectly—in the negative regulation of immediate-early gene expression. In ts48- and ts303-infected cells at the nonpermissive temperature, immediate-early polypeptides are overproduced in the presence of early polypeptides (Fig. 1 and 3). This observation indicates that early and delayed-early gene products have little direct effect on the regulation of immediate-early gene expression. Because late polypeptides are not made in cells infected with either class of ICP4 mutant, the possibility exists that late gene products may have an effect on immediate-early gene expression. The temperature shift-up experiment shown in Fig. 8 indicates that this may well be the case. After incubation of ts48-infected cells at 34°C for 3 and 6 h, immediate-early polypeptides ICP4 and ICP27 were overproduced upon shift-up to 39°C. The ability to overproduce immediate-early polypeptides upon shift-up, however, was lost after 6 h. This time coincides with the synthesis of viral DNA and late gene expression. Either the form of ICP4 in ts48-infected cells is stabilized with respect to thermal inactivation by events which occur in the cell at 6 h postinfection, or a late gene product is also involved with ICP4 in the negative regulation of immediate-early gene expression.
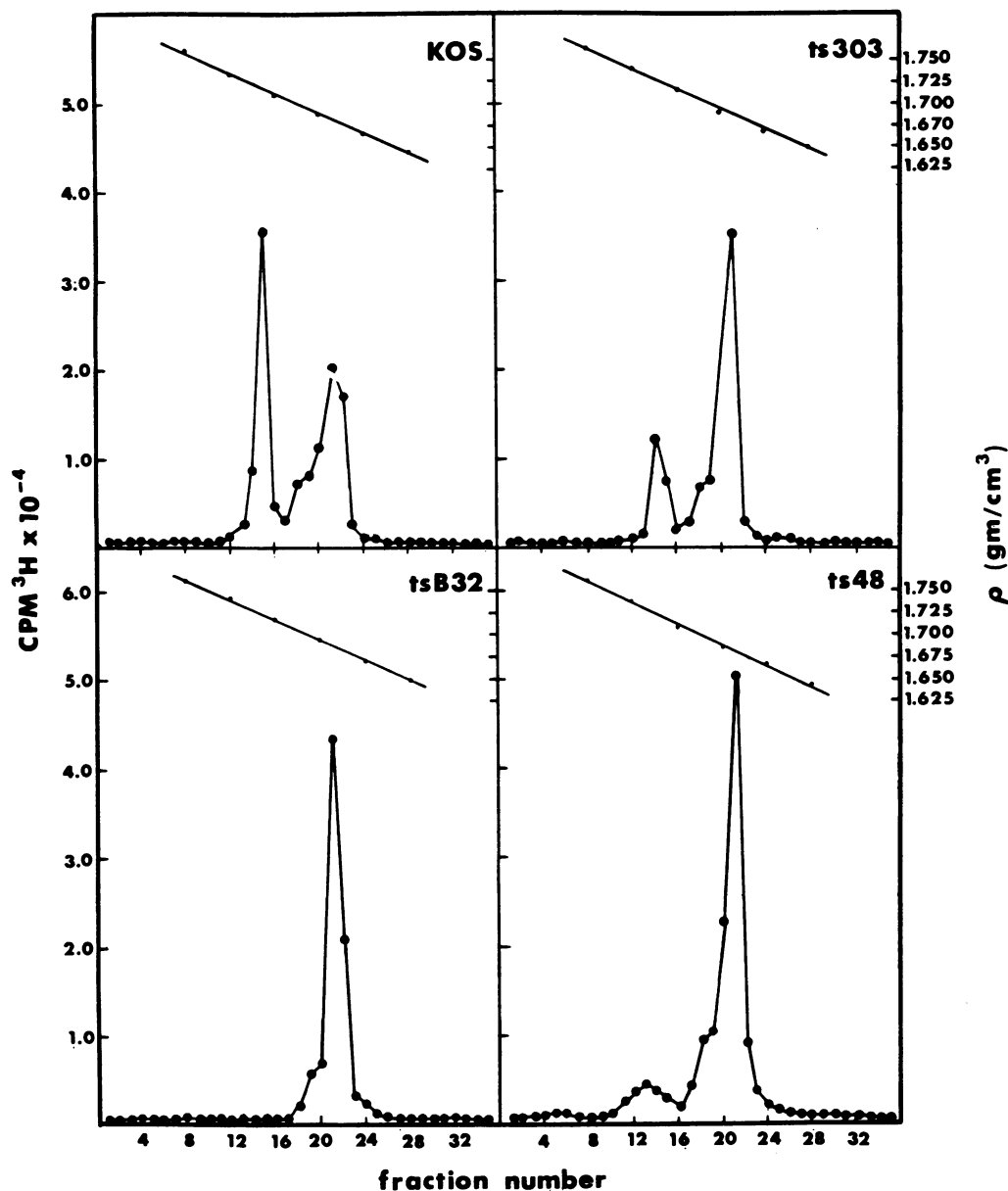
FIG. 6. Viral DNA phenotypes of *ts*48 and *ts*303 at 39.4°C. Infected cells were incubated at 39.4°C and labeled with 10 μCi of [³H]thymidine (Amersham) per ml from 4 to 24 h postinfection. DNA extracts from infected cells were prepared and analyzed by cesium chloride equilibrium centrifugation as described by Aron et al. (1), except that 200 μg of proteinase K per ml instead of pronase was used to deproteinize the DNA. Viral DNA peaked at a density of 1.725 g/cm³ and cellular DNA peaked at 1.685 g/cm³. The quantities of viral DNA synthesized by *ts*48- and *ts*303-infected cells compared with KOS-infected cells was in the range of 15 to 30% and 30 to 50%, respectively, in three separate tests. The quantities of viral DNA synthesized at 39.0°C were also with these ranges.

DISCUSSION

The hallmark of previously described *ts* mutants in complementation group 1-2 is the overproduction of immediate-early gene products and the failure to express all subsequent classes of genes. It has been established that ICP4 is the product of the gene defined by complementation group 1-2, and that ICP4 is directly responsible for both the activation of early genes and the negative regulation of immediate-early genes (9, 30–32). It has also been reported that some ICP4 mutants are able to induce the synthesis of slightly more early gene products under nonpermissive conditions than others (32); however, in no case did these levels reach that of

wild-type virus. In sharp contrast to other ICP4 mutants, the mutants described in this study are nearly as permissive as wild-type virus with respect to the synthesis of early gene products at 39°C, and both induce the synthesis of significant levels of viral DNA. Mutants *ts*48 and *ts*303 fail to complement each other and six other mutants which have been previously mapped to sequences within the gene for ICP4. Thus, the striking phenotypic characteristic of *ts*48 and *ts*303 is the elevated level of early gene expression under conditions which are nonpermissive for virus growth. Importantly, the phenotypic differences between *ts*48 and *ts*303 and other mutants in ICP4 is not a consequence of excessive leak by the former two mutants.
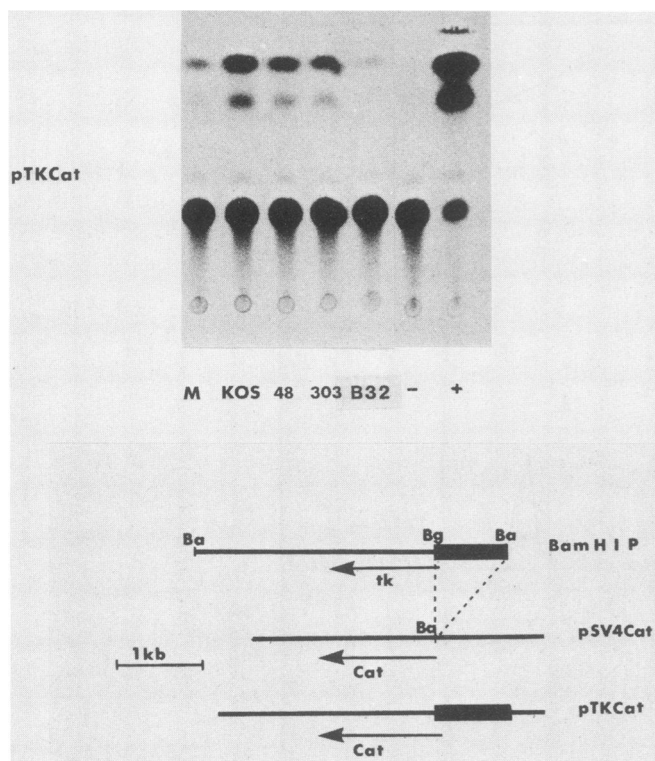
FIG. 7. Transient induction of pTKCat by KOS and *ts* mutants in ICP4. Approximately 10^6 CV-1 cells (passage 30) were transfected with 10 μg of pTKCat in duplicate as described (12). Four hours after addition of the DNA-CA^{2+} precipitate, monolayers were exposed to 15% glycerol in 1 × transfection buffer for 2.5 min. Monolayers were infected 3 h later as indicated at an m.o.i. of 20 PFU per cell and incubated at 39°C. Uninfected, transfected monolayers were treated similarly to determine the constitutive level of CAT expression from the *tk* promoter (lane M). Cells were harvested at 16 h postinfection, and sonicated extracts were prepared as described (12). The in vitro reaction for CAT contained the extract from 10^6 cells, 4 mM acetyl-coenzyme A, and 0.1 μCi of [^{14}C]chloramphenicol in 0.1 ml of 0.25 M Tris-hydrochloride (pH 7.8). The reaction was performed at 37°C for 1 h, at which time the chloramphenicol and the acetylated derivatives were extracted and separated by thin-layer chromatography as described (12). The position of untreated chloramphenicol (−) and the acetylated products (+) are indicated. The acetylated products (+) were generated by incubation with purified CAT enzyme (P-L Biochemicals, Milwaukee, Wis.). pTKCat was constructed by substituting the indicated *Bgl*II-*Bam*HI fragment for the simian virus 40 early region in pSV2Cat.

Mapping studies. The mutations conferring the temperature-sensitive phenotype on *ts*48 and *ts*303 were physically localized to the region of the genome encoding the 3′ end of the ICP4 gene. The *Hinc*II a fragment (map units 0.812 to 0.843) and the *Sau*3A b fragment (map units 0.835 to 0.845) (Fig. 2) both rescued *ts*48 and *ts*303 (Table 2). These fragments overlap between coordinates 0.835 and 0.843 and thus further localize the mutation to these sequences. The 3′ end of the ICP4 message is located 450 base pairs upstream from coordinate 0.835 (7). To date, no transcript has been identified immediately downstream from the 3′ end of the ICP4 message, and no transcripts have been identified which overlap the gene for ICP4. Taken together, the physical mapping and complementation data suggest that the mutations in *ts*48 and *ts*303 are localized within the 700-base pair sequence at the 3′ end of the ICP4 gene (map units 0.838 to 0.843). Dixon and Shaffer (9) have previously mapped five

other *ts* mutants of strain KOS in complementation group 1-2 to a fragment between map coordinates 0.831 (L-S junction) and 0.843 (the *Hinc*II site). These mutations must also map within the 3′ 700 base pairs of the ICP4 gene. It is of interest to note that all of the KOS mutants in group 1-2 isolated to date map within this region, regardless of the method of mutagenesis used in their induction: *ts*B2 (bromodeoxyuridine; 26), *ts*303 (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; 26), *ts*B21 (UV light) (26), *ts*B27, *ts*B28, and *ts*B32, the mutant used for comparison in this study (hydroxylamine) (5). By contrast, most mutants in group 1-2 derived from other strains map 5′ to this region (9, 32).

Early and late gene expression in *ts*48 and *ts*303. The levels of early and late polypeptides synthesized in *ts*48- and *ts*303-infected cells at the nonpermissive temperature were similar to levels observed in *ts*A24-infected cells under identical conditions (Fig. 1 and 4). Mutant *ts*A24 is defective in the 130,000-molecular-weight DNA-binding protein designated ICP8, a protein of the early kinetic class, and is wild type with respect to ICP4 function. Significant and approximately equal levels of early polypeptides were synthesized in *ts*48-, *ts*303-, and *ts*A24-infected cells at the nonpermissive temperature. Late polypeptides (i.e., ICP1-ICP2, ICP15, ICP19-ICP20, and gC) were not detected in cells infected with these three mutants. Consistent with the expression of early gene products in *ts*48- and *ts*303-infected cells at the nonpermis-
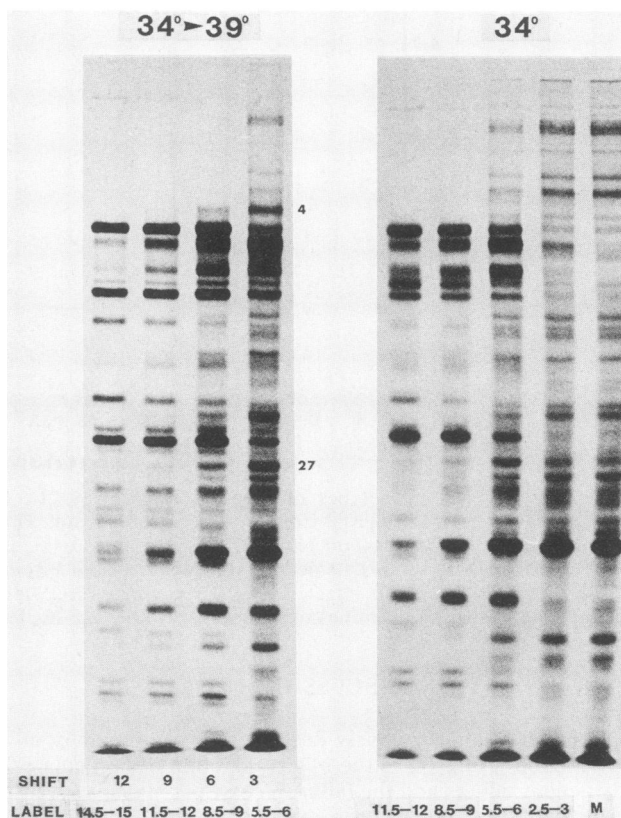

FIG. 8. Temperature shift-up experiments with *ts*48. *Ts*48-infected cells (m.o.i. = 20 PFU per cell) were incubated at 34°C and pulsed with [^{35}S]methionine (50 μCi/ml) at the indicated times, harvested, and prepared for SDS-PAGE. Replicate cultures were shifted to 39°C at the indicated times, incubated for 2.5 h, and then pulsed with [^{35}S]methionine for 30 min. An uninfected culture was labeled in the same manner (lane M). Cultures were harvested immediately after the pulse and prepared for SDS-PAGE.

sive temperature is the observation that significant levels of viral DNA were also synthesized (Fig. 6). Because different amounts of viral DNA were synthesized in cells infected with the various mutants used for comparison in this study (KOS, 100%; tsB32, 0%; ts48, 21%; and ts303, 41%), it is difficult to compare directly the levels of early gene expression induced by each mutant due to the inherent variation in the number of templates being transcribed. The transient expression-induction assay depicted in Fig. 7 shows the induction of CAT expression from an early promoter (tk) by the various mutants. Under these conditions an equal number of transcribing templates was present in all the cultures. Thus, the increased level of CAT activity induced by KOS compared with the uninfected culture is due to trans-induction of the tk-CAT construct. Trans-induction was not observed when transfected cells were infected with tsB32 at 39°C, which is indicative of the inability of the mutant form of ICP4 to activate the early promoter. In striking contrast, the mutant forms of ICP4 synthesized in ts48- and ts303-infected cells at 39°C were able to elevate the observed CAT activity to near wild-type levels. Additionally, the positive regulatory functions of the ICP4 synthesized in ts48- and ts303-infected cells at the nonpermissive temperature were sufficient to activate early genes, resulting in significant levels of viral DNA synthesis. Despite the synthesis of viral DNA, however, late gene products were not expressed. Therefore, early gene products alone are not sufficient for late gene expression; the continued expression of functional ICP4 is required.

Several explanations exist for the discrimination seen between expression of early and late genes in ts48- and ts303-infected cells compared with the observed block in early and late gene expression seen in other mutants in ICP4. A distinct form of ICP4, different from that required for early gene activation, may be required for late gene expression. ICP4 is present in a variety of phosphorylated forms in HSV-1-infected cells (28). It is possible that a specific phosphorylated form of ICP4 not produced in ts48- and ts303-infected cells at 39°C is essential for activation of late genes. Alternatively, late-gene expression may require an interaction between ICP4 and other HSV-1 gene products. This interaction may not occur in ts48- and ts303-infected cells at the nonpermissive temperature. Lastly, due to the enhanced transcription of immediate-early genes, sufficient cellular and viral components needed for transcription in general may not be available for transcriptional activation from the less active late promoters.

Immediate-early gene expression. Mutants ts48 and ts303 overproduce the immediate-early polypeptides at the nonpermissive temperature. ICP4 is overproduced in these mutants to the same extent as in tsB32-infected cells. ICP0 and ICP27 are overproduced to a lesser extent. At present it is not clear whether this reflects a difference inherent in the various immediate-early promoters or secondary effects having nothing to do with transcription. It has been postulated that ICP4 is involved in the negative regulation of immediate-early gene expression (9, 30). Therefore, this function of ICP4 is defective in ts48 and ts303. This defect was demonstrated in temperature shift-up experiments at early times after infection (Fig. 8). Shift-up at a time when ICP4 synthesis was no longer detectable (6 h postinfection) resulted in the increased synthesis of ICP4 and ICP27. Shift-up at later times (>6 h postinfection) did not result in an increase in immediate-early gene expression. The inability to reinitiate synthesis of ICP4 upon shift-up roughly coincides with DNA synthesis and the onset of late-gene expression.

After the onset of DNA synthesis, factors in addition to ICP4 may be required to limit the expression of immediate-early genes. These factors are, in all probability, not early functions since ts48 and ts303 make substantial levels of presumably wild-type early polypeptides. The findings presented in this study would therefore implicate a late function—acting in conjunction with, or in addition to, ICP4—in the negative regulation of immediate-early genes.

The activation of early and late genes and the negative regulation of immediate-early genes are all activities associated with ICP4. In addition, ICP4 appears to be involved in the discrimination between early and late genes. Due to the complexity of the HSV-1 regulatory cascade, the possible molecular mechanisms underlying the observed phenomena are numerous. Additional trans-induction experiments similar to those described herein and by others (14, 17) (Fig. 7) will be necessary to examine alternative regulatory mechanisms. Such experiments designed to better define the several possibilities raised by the phenotypes of ts48 and ts303 are currently in progress.

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